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Collagen type I degradation fragments act through the collagen receptor LAIR-1 to provide a negative feedback for osteoclast formation

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Running title: Regulation of osteoclasts by collagen degradation fragments

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ABSTRACT

The major organic component of bone is collagen type I. Osteoclasts are terminally differentiated multinucleated cells of hematopoietic origin that are essential for physiological development of bone and teeth. We examined if osteoclast differentiation from murine bone marrow precursors is affected by collagen type I, or by its degradation products produced by human recombinant cathepsin K. Osteoclasts formation was dose-dependently inhibited in the presence of full length collagen type I or its 30-75 kDa degradation products added to the osteoclast differentiation media for the duration of an experiment. Collagen degradation fragments signalled through SH-2 phosphatases, inhibiting calcium signalling and NFATc1 translocation in osteoclast precursors. Osteoclasts and their precursors expressed a collagen receptor of leukocyte receptor complex family, LAIR-1. Importantly, collagen fragments failed to inhibit osteoclast formation from LAIR-1 deficient murine bone marrow cells. This study demonstrates that collagen degradation fragments inhibit osteoclast formation acting through LAIR-1, providing a novel mechanism for the physiologically-relevant negative control of osteoclastogenesis.

Bone is a rigid organ; however, it is also highly dynamic due to its constant remodelling. Osteoclasts are the bone cells responsible for bone degradation. As tissue-specific cells, osteoclasts have targeted interactions with components of the extracellular matrix specific to bone. Since collagen type I is the most abundant protein in bone, osteoclast formation is likely regulated by the presence of collagen. In fact, one of the co-stimulatory receptors critically important for osteoclastogenesis, OSCAR, is now known to specifically bind to collagen [1]. Of interest, osteoclast functional activity results in the immediate change in the extracellular matrix environment, such as a decrease in the abundance of full length collagen and appearance of collagen degradation fragments. Thus, osteoclasts can be exposed to collagen type I in different forms – as a part of mature bone matrix to which osteoclasts attach, or as exposed fibrils or smaller degradation fragments generated during bone resorption. How these forms of collagen affect osteoclasts differentiation is not known.

Osteoclasts are multinuclear cells of hematopoietic origin. The differentiation from monocytes to osteoclasts is induced by cytokines such as receptor activator of nuclear factor kappa-B ligand (RANKL) and macrophage colony-stimulating factor (M-CSF), which bind to their respective cognate receptors, receptor activator of nuclear factor κ B (RANK) and colony stimulating factor 1 receptor (c-fms)[2]. Upon addition of these two factors to the culture medium, monocytes undergo commitment, followed by cell-cell fusion, and finally maturation of the giant polykaryons into specialized bone-resorbing osteoclasts. In addition to signalling induced by RANK and c-fms, successful osteoclastogenesis depends on the activation of co-stimulatory receptors, including OSCAR and TREM-2 [3]. These receptors require an adaptive protein, such as Fc γ R and DAP12, which stimulate phospholipase C (PLC) leading to the production of inositol-1,4,5- trisphosphate (IP₃) [4]. IP₃ activates the IP₃ receptor (IP₃R) resulting in Ca²⁺ release from the endoplasmic reticulum (ER) [5]. In committed osteoclast precursors calcium signaling is characterised by persistent oscillations of low magnitude [6]. These calcium oscillations lead to the activation of a number of downstream signaling molecules, including the phosphatase, calcineurin [7]. Calcineurin is critical for the activation of a key osteoclastogenic transcription factor, nuclear factor of activated T-cells c1 (NFATc1). Inactive NFATc1 exists in the cytosol in a hyperphosphorylated form. Calcineurin dephosphorylates NFATc1, allowing it to translocate to the nucleus, where it regulates the expression of osteoclastic genes, including cathepsin K and TRAP [8].

Another collagen receptor, LAIR-1, from the same family of leukocyte receptor complex has been discovered in immune cells [9], and was implicated in osteoclast regulation [10]. In contrast to OSCAR that signals through Fc γ R containing immunoreceptor tyrosine-based activation motif (ITAM), LAIR-1 has an immunoreceptor tyrosine-based inhibition motif (ITIM) [9]. ITIM-containing receptors can inhibit calcium signalling by compartmentalizing and activating phosphatases, such as SH2 domain-containing protein-tyrosine phosphatases 1 and 2 (SHP1 and SHP2) and the SH2 domain-containing inositol phosphatase 2 (SHIP2) [11, 12], and the inhibitory C-terminal Src kinase (Csk), in the case of LAIR-1 [13, 14]. While OSCAR is an osteoclast stimulatory receptor, it can be hypothesized that activation of antagonistic LAIR-1 will lead to inhibition of osteoclast formation. However, the physiological role of such inhibition remains unclear.

The objective of this study was to assess the effects of collagen and its degradation fragments on osteoclastogenesis and to examine the roles of OSCAR and LAIR-1 in mediating these effects.

Results

To examine the effect of collagen on osteoclast formation, we coated the tissue culture plates with collagen type I at different densities or applied the corresponding concentrations as part of the culture medium and examined osteoclast differentiation (Fig. 1). On collagen type I-coated plates, osteoclastogenesis was inhibited on day 5 by the highest concentration of collagen, but was recovered by day 7 (Fig 1A,C). When collagen type I was added in solution, it induced progressive dose-dependent inhibition of osteoclast formation (Fig 1B,D). The few osteoclasts that were formed in the presence of high media concentrations of collagen type I ($50 \mu\text{gml}^{-1}$) were considerably smaller (Fig. 1E). Thus, the addition of collagen type I to the apical surface of the osteoclast precursors produced a strong inhibition of osteoclast differentiation.

During bone resorption, osteoclasts transcytose degraded collagen fragments [15]. Therefore, we hypothesized that collagen degradation fragments facilitate physiologically-relevant negative feedback on osteoclast differentiation. To directly test if collagen type I degradation fragments have osteoclast inhibitory activity, we simulated osteoclastic digestion of collagen type I using recombinant cathepsin K [16]. *In tube* digestion of collagen type I resulted in the time-dependent generation of multiple smaller fragments (Fig. 2A). We tested the effect of the complete digest generated by 1.5 h exposure of collagen type I to $0.2 \mu\text{M}$ of active cathepsin K, on osteoclast formation. Application of collagen fragments inhibited osteoclast formation in a concentration dependant manner (Fig. 2B). Compared to full length collagen added in solution, the effect of collagen fragments was apparent earlier (day 5 compared to day 7 of osteoclastogenesis) and at lower amounts ($10\text{-}30 \mu\text{g/ml}$ compared to $50 \mu\text{g/ml}$ for full length collagen type I) We tested three control buffers representing the digestion solution without cathepsin K (Ctl 1, similar to solution containing full length collagen type I), without collagen type I (Ctl 2) and without cathepsin K and collagen type I (Ctl 3). None of the controls significantly affected osteoclastogenesis on day 5 and the effects of the digest were significantly different from all corresponding controls (Fig. 2 B,C). We further separated the fragments using reverse phase chromatography (Fig. 3A). The fraction containing collagen type I fragments of $30\text{-}75 \text{ kDa}$ demonstrated osteoclast inhibitory activity, while the fractions containing fragments smaller than 20 kDa , or larger than 75 kDa , as well as control solutions did not affect osteoclastogenesis on day 5 (Fig. 3 B, C). Thus, collagen type I degradation fragments that were $30\text{-}75 \text{ kDa}$ in size strongly inhibit osteoclast formation.

We next examined if treatment with collagen type I fragments (CF) affects calcium/NFATc1 signaling [17]. Osteoclastogenesis from bone marrow precursors was induced in the absence or presence of CF and after 3 days, NFATc1 localization was monitored by immunofluorescence. NFATc1 nuclear translocation was significantly reduced in monocytes treated with CFs (Fig. 4A). Since NFATc1 translocation is regulated by the calcium-dependent phosphatase calcineurin, we next investigated if characteristic calcium oscillations in osteoclast precursors [6] were negatively affected by CF. Cytosolic free calcium ($[\text{Ca}^{2+}]_i$) in fura2 loaded osteoclast precursors was examined after 3 days of culture with or without CFs (Fig. 4B,C). Addition of CFs significantly reduced the proportion of cells exhibiting calcium transients, but did not significantly affect the amplitude or periodicity of the calcium oscillations in the responding cells (Fig. 4D). We examined the role of the negative upstream regulators of PLC γ -dependent calcium signaling, phosphatases SHP1/2 and SHIP2, using pharmacological inhibitors.

Osteoclastogenesis from bone marrow precursors was induced with or without CFs, in the absence or presence of SHP1/2 or SHIP2 inhibitors, and osteoclast formation after 5 days of culture was assessed (Fig. 4E,F). Inhibition of SHP1/2 or SHIP2 significantly reduced the number of osteoclasts formed in the positive control, emphasizing the importance of these phosphatases for osteoclastogenesis. Nevertheless, no additional osteoclast-inhibitory effect of CFs was observed when either SHP1/2 (Fig. 4E) or SHIP2 (Fig. 4F) was inhibited. These data suggest that CFs signalling requires the action of SH-2 phosphatases, which inhibit calcium signaling and subsequently NFATc1 translocation in osteoclast precursors during differentiation.

Signaling induced by CF is consistent with the involvement of ITIM-containing receptors of leukocyte receptor complex, such as a known collagen receptor, LAIR-1 [9]. We examined gene expression of *Oscar* and *Lair-1* during osteoclastogenesis (Fig. 5A,B). While *Oscar* expression increased dramatically (Fig. 5A), the expression of *Lair-1* was maintained during osteoclast formation (Fig. 5B). We confirmed that LAIR-1 protein was expressed in osteoclasts at the levels comparable to those in macrophages and K562 human myelogenous leukemia cells, but not in HEK293 human embryonic kidney cells which we used as a negative control (Fig. 5C). Immunofluorescence confirmed localization of both OSCAR and LAIR-1 in monocytes and osteoclasts (Fig. 5D, E). Thus, ITIM-containing inhibitory collagen receptor LAIR-1 is expressed on osteoclasts and their precursors together with a stimulatory ITAM-containing collagen receptor, OSCAR.

To directly examine the role of LAIR-1 in mediating CF-induced inhibition of osteoclastogenesis, we used monocytic precursors from animals with global knockout of *Lair-1* [18]. We assessed osteoclastogenic potential of spleen monocytes from *Lair-1*^{-/-} animals and their wild type (WT) littermates and observed a significant increase in osteoclast differentiation from precursors obtained from *Lair-1*^{-/-} mice (Fig. 6A). We further studied the effect of full length collagen type I (FL) or collagen type I degradation fragments (CF) on osteoclast formation from spleen precursors of *Lair-1*^{-/-} and WT mice. We observed that while both FL and CF inhibited osteoclast formation in WT cultures, only FL was effective in *Lair-1*^{-/-} cultures, while CF failed to induce osteoclast inhibition in the absence of LAIR-1 (Fig. 6B). Consistent with these observations, NFATc1 nuclear translocation was inhibited by CF in WT cultures, but not in *Lair-1*^{-/-} cultures (Fig. 6C), and osteoclast size was unaffected by CF in the absence of LAIR-1 (Fig. 6D). These data demonstrate that LAIR-1 is critical for the osteoclast-inhibitory effects of collagen type I degradation fragments, but not for the action of full length collagen type I.

Discussion

We have found that osteoclastogenesis is inhibited by full length collagen type I, and to a higher degree by collagen type I degradation fragments added to the apical cell surface. This inhibition was dose dependent and was most prominent when collagen fragments of 30-75 kDa in size were used. Collagen type I fragments were found to inhibit calcium oscillations and NFATc1 nuclear translocation in osteoclast precursors. Importantly, the absence of the receptor LAIR-1 interfered with the ability of collagen degradation fragments to inhibit osteoclast formation but did not affect inhibition induced by full length collagen type I. These data suggest that osteoclastic degradation of collagen type I during resorption provides a potent negative feedback that limits osteoclast formation and function.

The osteoclast inhibition by collagen fragments is similar to the osteoclast inhibitory effects of fibrillin-1 degradation fragments we reported previously [19]. In line with the previous study, we demonstrated that collagen type I proteolytically processed during osteoclastic resorption results in formation of multiple degradation products. These fragments produced by collagen type I *in tube* digestion with cathepsin K were similar to those observed during bone resorption [20, 21]. The fragments between 30-75 kDa in size added to the apical cell surface were most effective in inhibiting osteoclastogenesis. Previously cathepsin K-generated collagen fragments were also demonstrated to inhibit osteoclast resorptive activity [22]. The effects we observed are likely relevant to previously described transcytosis of matrix degradation products from basal surface where resorption occurs to the apical surface for further removal [23]. In our experiments, immobilized collagen exhibited very low inhibitory activity, even though it can be expected that it is equally processed by the osteoclasts. It is possible that lower levels of collagen fragments were produced under these conditions, or that they were diluted during media change. Taken together, our study demonstrates that negative feedback from effective osteoclastic resorption to the formation of new osteoclasts is generated by degradation fragments of multiple ECM proteins, which implies the level of redundancy observed in a critical process.

Our data indicate that osteoclast inhibition by collagen fragments is mediated by LAIR-1. We have demonstrated that both osteoclast precursors and mature osteoclasts retain the expression of LAIR-1. Importantly, inhibition of osteoclastogenesis by collagen fragments was not observed when monocytes deficient in LAIR-1 were tested. Moreover, we have found that inhibition of osteoclastogenesis by collagen fragments required the action of SH2-containing phosphatases, which are known to be involved in ITIM-induced signaling [24]. Inhibition of calcium signaling and NFATc1 translocation by collagen degradation fragments is also consistent with ITIM-mediated inhibition of OSCAR-induced ITAM-mediated signaling [25]. LAIR-1 and OSCAR were previously suggested to co-express in osteoclasts [1], however it was unclear how the opposing actions of LAIR-1 and OSCAR can be coordinated during osteoclast formation. We propose that during the osteoclast polarization the OSCAR receptor is located on the basal surface of the osteoclast and is stimulated by the full-length collagen present in the bone matrix. In contrast, we suggest that LAIR-1 receptor is localized on the apical surface and where it is stimulated by the degradation fragments produced during resorption. Therefore, OSCAR and LAIR-1 play distinct roles during osteoclastogenesis, where OSCAR provides identification of a correct substrate for differentiating precursors, while the action of LAIR-1 is required for fine-tuning osteoclast formation to the current rate of bone resorption.

Taken together, our data suggest that the negative feedback produced by collagen (and fibrillin-1) degradation fragments on osteoclast formation represents an important and novel regulatory pathway for fine-tuning osteoclastogenesis. The implication of a novel osteoclast receptor, LAIR-1, in these effects provides a new potential therapeutic target for development of osteoclast-limiting drugs.

Experimental Procedures

Animals – All experiments were approved by the Animal Care Committee at the McGill University and conformed to the ethical guidelines of the Canadian Council on Animal Care. Animals had unrestricted access to food and

water, and were on a 12-hour alternating light and dark cycle. *LAIR-1*^{-/-} were generated as previously describe[18]. Male 7-20 week old wild type littermates or *Lair-1*^{-/-} mice were used for the experiments.

Osteoclasts cultures– Osteoclasts were obtained using a previously published protocol [26]. Briefly, mouse bone marrow and spleen cells were isolated. The cell suspension was centrifuged, and the pellet treated with red blood cell lysis buffer (Sigma Aldrich Ltd; R7757). The remaining marrow cells were resuspended in medium supplemented with MCSF and incubated overnight. Non-adherent cells were collected the next day and plated with MCSF and RANKL, media was changed every 2 days and osteoclasts were generally observed on day 5-7 of culture. The osteoclast cultures were exposed to collagen type I or its degradation fragments for the duration of the experiment.

Quantification of osteoclasts – Mature osteoclast cultures were fixed using 10% formalin in PBS pH 7.4, for 8–10 min at room temperature and stained for tartrate resistant acid phosphatase (TRAP) using a commercial kit (TRAP, Sigma: 387A-KT) as reported previously [26].

Collagen digestion using cathepsin K – Collagen type I (0.4 mgmL⁻¹) (isolated from mouse tails) was incubated with recombinant human cathepsin K (0.2 µM) in 100 mM sodium acetate buffer, pH 5.5, containing 2.5 mM DTT and 2mM EDTA. Cathepsin K was produced in *Pichia pastoris* as previously described [27]. Digestion was performed *in tube* using protocol previously presented [16]. Collagen digestion was performed at 32 °C in the presence of 0.15% (w/v) chondroitin 4-sulfate[16]. The digestion reaction was stopped by the addition of 10 µM E64 after 1.5 h. The samples were subjected to SDS-polyacrylamide electrophoresis on 4–20% Tris/glycine gels that were subsequently analysed with silver staining.

Reverse phase chromatography of the digested collagen– After *in tube* digestion by cathepsin K, 10 ml collagen fragments were injected in a sep-pak Plus C8 column (Waters Corp: WAT036775), preloaded with a solution of 0.1% of formic acid and 2% acetonitrile in double distilled water. The fragments were eluted using 5 ml each of solutions of 0, 25, 50, 75 and 100% of acetonitrile with 0.1% of formic acid. The fractions were resolved using a 4–20% Tris/glycine gels analysed with silver staining.

RNA isolation, qRT PCR – Total RNA was isolated from bone marrow- and spleen-derived osteoclasts with the RNeasyMini Kit (Qiagen Inc.:74106, Toronto, ON, Canada). RNA and cDNA concentration were quantified with the Quanti- iT™ instrument (Invitrogen: Q32860, Burlington, ON, Canada). Reverse-transcription of 0.75 ng RNA was performed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Fisher Scientific; Nepean, ON, Canada). Real-time PCR was performed using a 7500 Applied Biosystems Instrument, the Taq-Man Universal PCR Master Mix (Applied Biosystems, Burlington, ON, Canada). The TaqMan probes were: *Oscar* (Mm00558665_m1), *Lair-1* (Mm01332313_g1) and Glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*; Mm99999915_g1) used as the endogenous control. MCSF only treated bone marrow precursors were used as the calibrator.

Immunoblotting – Whole cell lysates were prepared as described previously [28]. Briefly, the cells were lysed using a RIPA buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 1 mM EDTA, 10 mgmL⁻¹ aprotinin, 1 mgmL⁻¹ leupeptin, 0.1 M phenylmethylsulfonyl fluoride, 0.5 M sodium fluoride and 1 mM sodium orthovanadate). Protein concentrations were determined using a Quant-iT™ protein assay kit (Invitrogen). Proteins (50 µg) were

taken and 5x SDS sample buffer was added, separated by 7.5% gel electrophoresis under reducing conditions, transferred to a nitrocellulose membrane (Bio-Rad, #162-0115; 0.45 μ m) using 10 mM sodium borate, pH 8.9. Membranes were blocked in 5% non-fat milk in TBST buffer (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.05% Tween 20) for 1 h at 4°C and then washed and incubated overnight at 4°C with primary antibodies for LAIR-1 (1:500, 16-3051-82, eBioscience). The following day, membranes were washed in TBST and incubated with horseradish peroxidase-conjugated secondary antibodies (1:1000 dilution rabbit anti-Armenian Hamster; ab5745 Abcam) for 1 hr at room temperature, and finally visualized using a chemiluminescence substrate (Thermo Scientific, #32106).

Immunofluorescence– Immunostaining for NFATc1, OSCAR and LAIR-1 was performed as described previously [29]. Briefly, osteoclasts on glass coverslips were fixed with 10% formalin, incubated with mouse anti-NFATc1 antibody (1:100, Santa Cruz Biotechnology; #SC-7294), OSCAR (1:200, SC-34237, Santa Cruz), LAIR-1 (1:200, 16-3051-82, eBioscience) followed by a biotinylated goat anti-mouse IgG (1:200, Invitrogen; # A10519) and Alexa-Fluor- 488-conjugated streptavidin (1:500, Invitrogen; #S-11223) for NFATc1 and OSCAR. First antibody of LAIR-1 was followed by Rhodamine conjugated goat anti-mouse IgG (1:1000, Santa Cruz; SC-3932). Nuclei were counterstained using 49,6-diamidino-2-phenylindole dihydrochloride (DAPI; 1:10,000, Invitrogen; #D1306). For each experiment, five random fields of view per condition were imaged.

Microspectrofluorometry – Cells plated onto glass-bottom 35 mm dishes (MatTek.) were loaded with Fura2-AM (Invitrogen; F1221) at room temperature for 30 min and washed twice with physiological solution. A final volume of 1 mL physiological solution was added and cells were acclimatized for 10 min. Changes in $[Ca^{2+}]_i$ were measured for 120 s, at a sampling ratio of 2 images per second using a Nikon T2000 fluorescent inverted microscope. The excitation wavelength was alternated between 340 and 380 nm using ultra high-speed wavelength switching illumination system (Lambda DG-4, Quorum Technologies). Regions of interest were manually defined and the ratio of the fluorescence emission at 510 nm, flowing 340 and 380 nm excitation was calculated and exported using imaging software (Volocity, Improvision). All data were imported into an excel spreadsheet for subsequent analysis. Calcium recordings were characterized using MATLAB algorithm previously described [30]. The following parameters were obtained for subsequent statistical analysis: average calcium ratio (average $[Ca^{2+}]_i$ (340/380 ratio)), % responders, amplitude, % oscillatory responders and magnitude of oscillatory peaks.

Statistics – Data are presented as means \pm standard error of the mean (SEM) with sample size (n) indicating the number of independent experiments. Data obtained using osteoclast precursors isolated from a single mouse were considered as one independent experiments. At least three independent experiments were performed for each assay. Differences were assessed by ANOVA with Bonferroni post-test or Student's t-test when appropriate and accepted as statistically significant at $p < 0.05$.

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Author contributions: Study conception: IB, SVK. Study design: IB, JSM, DB, YAS, AM. Acquisition of data: IB. Analysis and interpretation of data: IB, JSM, SVK. Drafting of Manuscript: IB, SVK. All authors contributed to the critical revision and approval of the final manuscript.

Figure legends

Figure 1. Collagen type I inhibits osteoclastogenesis. **A-D**) Bone marrow cells were plated on tissue culture plates coated with 0-50 $\mu\text{g}/\text{cm}^2$ collagen type I (**A,C**) or were plated on uncoated tissue culture plates and 0-50 $\mu\text{g}/\text{cm}^2$ collagen type I was added to the solution (**B,D**). Differentiation was induced with MCSF (50 ng/ml) and RANKL (50 ng/ml) and after 5 days (**A,B**) or 7 days (**C,D**) the samples were fixed and stained for TRAP. Data are average osteoclast (Oc) numbers, means \pm SEM, $n = 6-7$ independent experiments, $*p < 0.05$ indicates statistical significance compared to positive control (0 $\mu\text{g}/\text{cm}^2$ collagen type I) by ANOVA with Bonferroni post-test. **E**) Representative images of cells cultured for 7 days without RANKL (negative control), with RANKL but without collagen type I (positive control), on plates coated with 50 $\mu\text{g}/\text{cm}^2$ collagen type I, or in the presence of 50 $\mu\text{g}/\text{cm}^2$ collagen type I in the culture medium.

Figure 2. Osteoclastogenesis is strongly inhibited by collagen type I degradation fragments. **A**) *In tube* digestion of collagen type I was performed in the presence of 0.1-0.2 μM of Cathepsin K (Cts K). The reaction was stopped after 0.5-1.5 h with 10 μM E64 and collagen fragmentation pattern was visualized on silver-stained SDS page gel. **B, C**) Bone marrow cells were treated with media containing MCSF and RANKL only (positive control, PC) or MCSF, RANKL and either collagen type I fragments (CF), or the control solutions that were the same as the solution used for collagen type I digest but without cathepsin K (Ctl 1); without collagen type I (Ctl 2) or without collagen type I and cathepsin K (Ctl 3). After 5 days, the samples were fixed and stained for TRAP. **B**) Average numbers of osteoclasts observed in cultures treated with 5-30 $\mu\text{g}/\text{ml}$ of collagen type I fragments or with control solutions in amounts equivalent to those added with 20 or 30 $\mu\text{g}/\text{ml}$ CF. Data are means \pm SEM, normalized to positive control (red dotted line), $n = 8$ independent experiments; $*p < 0.05$, $**p < 0.01$ indicate statistical significance compared to PC by ANOVA with Tukey post-test; $\#p < 0.05$ indicate statistical significance for CF- and control solution-treated samples by Student's t-test. **C**) Representative images of osteoclasts formed in positive control (PC), in the presence of 30 $\mu\text{g}/\text{ml}$ collagen type I degradation fragments (CF) or control solution without cathepsin K (Ctl 1).

Figure 3. Collagen type I fragments between 30-75 kDa have the strongest inhibitory effect. **A**) Representative silver-stained SDS page gradient gel demonstrating the separation of collagen fragments with acetonitrile (ACN) gradient. Elutions combined to test their effect on osteoclastogenesis are labeled as 1 (>75 kDa), 2 (30-75 kDa) and 3 (<20 kDa). **B**) Average numbers of osteoclasts observed in cultures treated for 5 days with collagen type I fragments of different molecular weight or with control solutions in equivalent amounts. Data are means \pm SEM,

normalized to positive control (red dotted line), $n = 3$ independent experiments; $*p < 0.05$ indicates statistical significance compared to PC, by ANOVA with Bonferroni post-test. **C)** Representative images of osteoclasts formed in the presence of collagen type I fragments of different molecular weight.

Figure 4. Signalling events affected by collagen type I fragments. Bone marrow cells were treated with media containing MCSF and RANKL only (positive control, PC) or MCSF, RANKL and collagen type I fragments (CF, 25 $\mu\text{g/ml}$). **A)** The cultures were fixed on day 3 and NFATc1 localization was examined by immunofluorescence. *Left:* representative images of NFATc1 (green) and DAPI (blue) in osteoclast precursors from negative control (NC, cells treated with MCSF only), positive control and CF-treated cultures. *Right:* average percentage of osteoclast precursors exhibiting nuclear localization of NFATc1. Data are means \pm SEM, $n = 3$ independent experiments, $***p < 0.001$ indicates statistical significance by Student t-test. **B,C)** Representative traces of calcium oscillations observed on day 3 in positive control and collagen type I fragments-treated cultures. **D)** Calcium traces were quantified and presence of responding cells, proportion of oscillatory responses in responding cells, and the magnitude of oscillations were assessed in positive control or CF-treated cultures. Data are means \pm SEM, $n = 8$ independent experiments, $*p < 0.05$ indicates statistical significance by Student t-test. **E)** The cultures were treated with the inhibitor of Shp1/2 (20 μM) for 3 h on day 1 and 3, and fixed on day 5. Average numbers of osteoclasts formed in different conditions. **F)** Inhibitor for Ship 2 (1 μM) was added to the culture medium and the samples were fixed on day 5. For E and F, data are means \pm SEM, $n = 3$ independent experiments, $*p < 0.05$ indicates statistical significance by Student t-test.

Figure 5. Expression of collagen receptors in osteoclasts and their precursors. **A, B)** Relative gene expression of *Oscar* and *Lair-1* in cultures treated for 5 days with MCSF only (NC) or with MCSF and RANKL (PC). For A and B, data are means \pm SEM, normalized to endogenous control, with one of the positive control samples used as a calibrator, $n = 3-6$ independent experiments, $***p < 0.001$ indicates statistical significance by Student t-test. **C)** Protein expression of LAIR-1 in negative control cultures, differentiated osteoclasts, K562 cells and HEK293 cells assessed by immunoblotting. **D, E)** Representative immunofluorescence images for OSCAR (**D**, green) and LAIR-1 (**E**, red) and DAPI (blue) in negative control cultures (NC) and in osteoclasts (PC). White outlines indicate the borders of an osteoclast.

Figure 6. LAIR-1 is required for osteoclast inhibition by collagen type I fragments. Monocytic spleen precursors from *Lair1*^{-/-} animals (KO) and wild type littermates (WT) were treated with media containing MCSF and RANKL. **A)** Samples were fixed after 9 days of culture and average osteoclast numbers were assessed. Data are means \pm SEM, $n = 4-6$ mice per condition; $*p < 0.05$ indicates statistical significance compared to WT, by Student t-test. **B,C)** *Lair1*^{-/-} (KO) or WT spleen precursors were treated with MCSF, RANKL and control solution (Ctl 1), full length collagen (FL, 50 $\mu\text{g/ml}$), or collagen type I fragments (CF, 25 $\mu\text{g/ml}$). **B)** Average osteoclast numbers after 9 days of culture normalized to positive control (PC). **C)** Samples were fixed after 5 days of culture and NFATc1 localization was assessed. For B and C, data are means \pm SEM, $n = 3-4$ mice per condition, $*p < 0.05$ indicates statistical

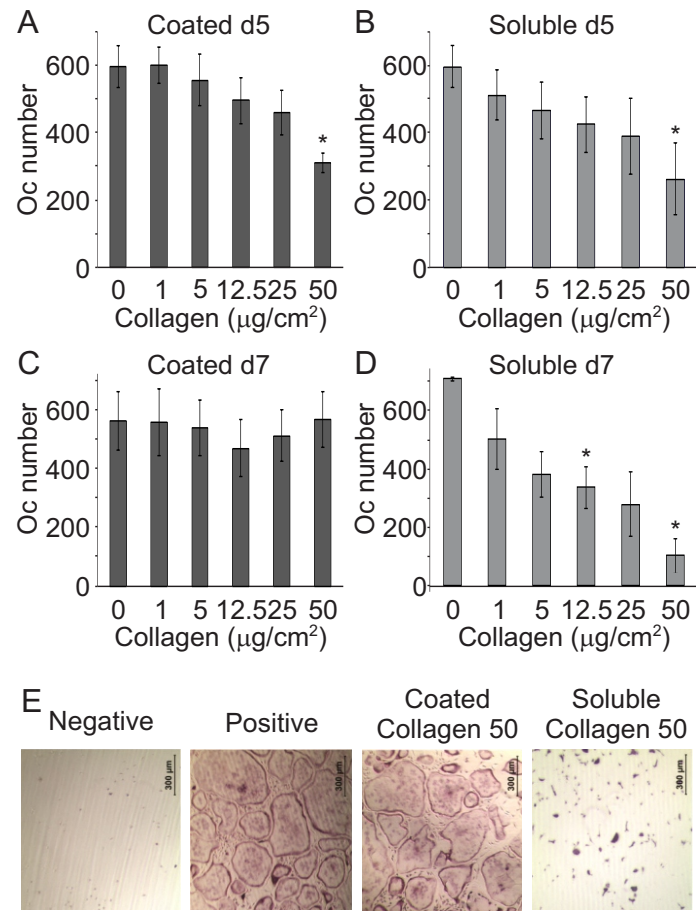
significance compared to PC of the same genotype, #p<0.05 indicates statistical significance compared to WT with the same treatment, by Student t-test. **D)** Representative images of osteoclast observed in control cultures and in the presence of FL and CF in WT and KO animals.

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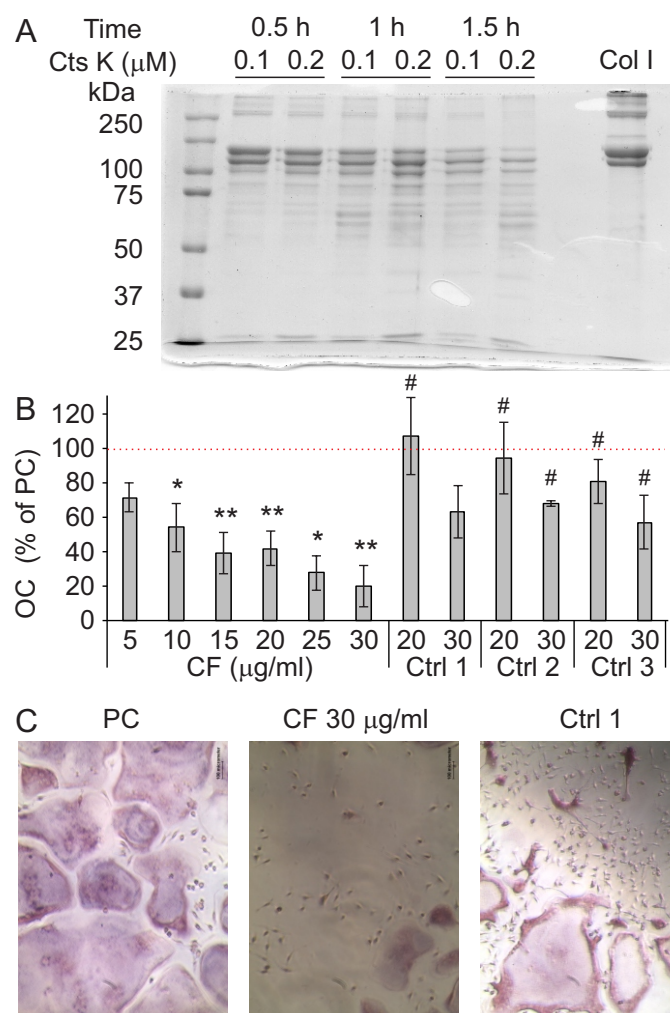
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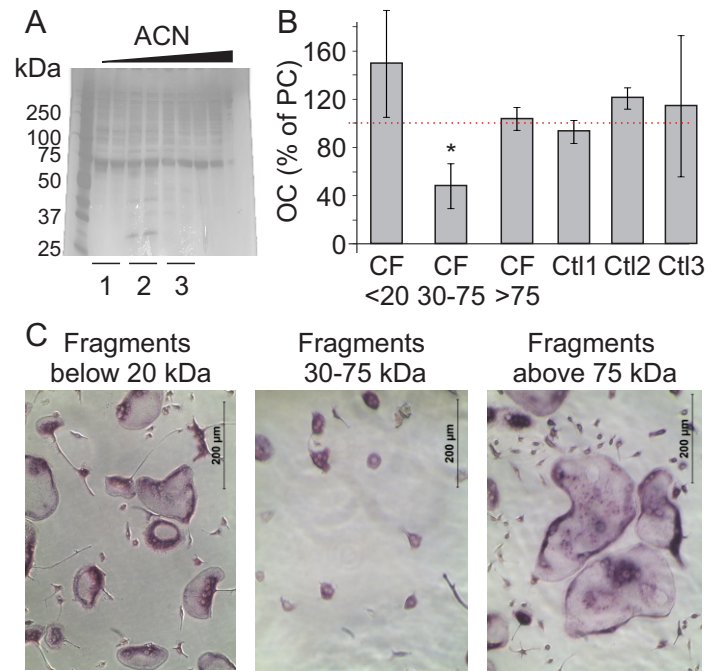
Boraschi-Diaz et al. Fig 1



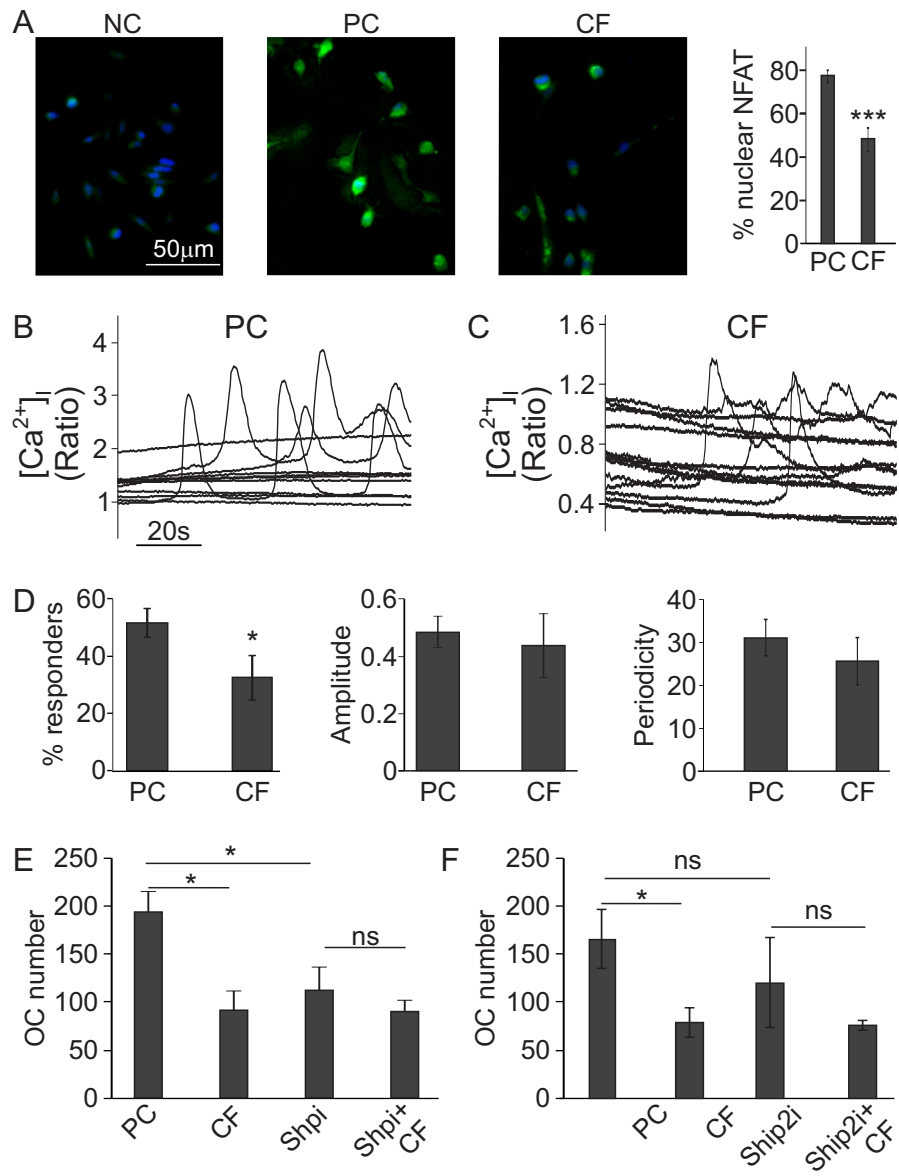
Boraschi-Diaz et al. Fig 2



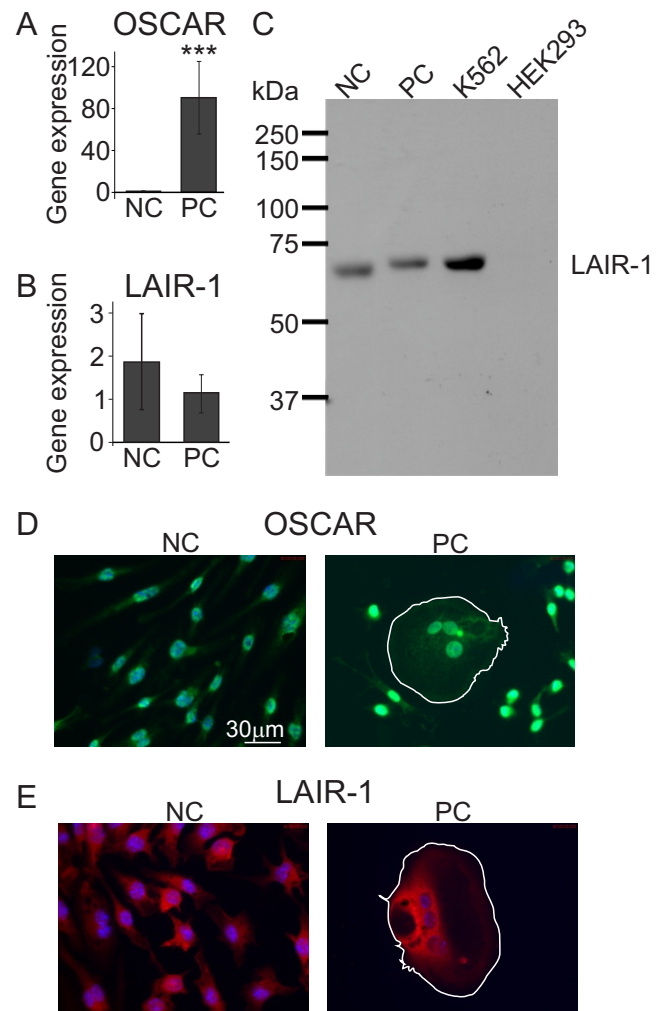
Boraschi-Diaz et al. Fig 3



Boraschi-Diaz et al. Fig 4



Boraschi-Diaz et al. Fig 5



Boraschi-Diaz et al. Fig 6

